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(54) **Title:** CONJUGATES OF HYDROXYETHYL STARCH AND ERYTHROPOIETIN

(57) **Abstract:** The present invention relates to conjugates of hydroxyethyl starch and erythropoietin, wherein these conjugates comprise a linking compound which is covalently linked to erythropoietin and covalently linked to hydroxyethyl starch. The present invention also relates to the method of producing these conjugates, and their use.

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Conjugates of Hydroxyethyl Starch and Erythropoietin

The present invention relates to conjugates of hydroxyethyl starch (HES) and erythropoietin (EPO), wherein these conjugates comprise a linking compound which is covalently linked to EPO and covalently linked to HES. The present invention also relates to the method of producing these conjugates and their use.

EPO is a glycoprotein hormone necessary for the maturation of erythroid progenitor cells into erythrocytes. In human adults, it is produced in the kidney. EPO is essential in regulating the level of red blood cells in the circulation. Conditions marked by low levels of tissue oxygen provoke an increased biosynthesis of EPO, which in turn stimulates erythropoiesis. A loss of kidney function as it is seen in chronic renal failure, for example, typically results in decreased biosynthesis of EPO and a concomitant reduction in red blood cells.

Erythropoietin is an acid glycoprotein hormone of approximately 34,000 D. Human erythropoietin is a 166 amino acid polypeptide that exists naturally as a monomer (Lin et al., 1985, PNAS 82, 7580-7584, EP 148 605 B2, EP 411 678 B2). The identification, cloning and expression of genes encoding erythropoietin are described, e.g., in U.S. Patent 4,703,008. The purification of recombinant erythropoietin from cell culture medium that supported the growth of mammalian cells containing recombinant erythropoietin plasmids, for example, is described in U.S. Patent 4,667,016.

It is generally believed in this technical field that the biological activity of EPO in vivo mainly depends on the degree of sialic acids bound to EPO (see e.g. EP 428 267 B1). Theoretically, 14 molecules of sialic acid can be bound to one molecule EPO at the terminal ends of the carbohydrate side chains linked to N- and O-glycosylation sites. Highly sophisticated purification steps are necessary to obtain highly sialylated EPO preparations.

For further detailed information on erythropoietin see Krantz, Erythropoietin, 1991, Blood, 77(3):419-34 (Review) and Cerami, Beyond erythropoiesis: novel applications for recombinant human erythropoietin, 2001, Semin Hematol., (3 Suppl 7):33-9 (Review).

A well-known problem with the application of polypeptides and enzymes is that these proteins often exhibit an unsatisfactory stability. Especially, erythropoietin has a relatively short plasma half live (Spivak and Hogans, 1989, Blood 73, 90; McMahon et al., 1990, Blood 76, 1718). This means that therapeutic plasma levels are rapidly lost and repeated intravenous administrations must be carried out. Furthermore, in certain circumstances an immune response against the peptides is observed.

It is generally accepted that the stability of polypeptides can be improved and the immune response against these polypeptides is reduced when the polypeptides are coupled to polymeric molecules. WO 94/28024 discloses that physiologically active polypeptides modified with polyethyleneglycol (PEG) exhibit reduced immunogenicity and antigenicity and circulate in the bloodstream considerably longer than unconjugated proteins, i.e. have a reduced clearance rate.

However, PEG-drug conjugates exhibit several disadvantages, e.g. they do not exhibit a natural structure which can be recognized by elements of in vivo degradation pathways. Therefore, apart from PEG-conjugates, other conjugates and protein polymers have been produced. A plurality of methods for the cross-linking of different proteins and macromolecules such as polymerase have been described in the literature (see e.g. Wong, Chemistry of protein conjugation and cross-linking, 1993, CRCS, Inc.).

WO 03/074087 relates to a method of coupling proteins to a starch-derived modified polysaccharide. The binding action between the protein and the polysaccharide, hydroxyalkyl starch, is a covalent linkage which is formed between the terminal aldehyde group or a functional group resulting from chemical modification of said terminal aldehyde group of the hydroxy alkyl starch molecule, and a functional group of the protein. As reactive group of the protein, amino groups, thio groups and carboxyl groups are disclosed. Specifically, WO 03/074087 is silent on the possibility of coupling hydroxy alkyl starch to a carbohydrate moiety of the protein. Moreover, whilst a vast variety of possibilities of different linkages is given in the form of many lists, including different functional groups,

theoretically suitable different linker molecules, and different chemical procedures, the working examples describe only two alternatives: first, an oxidized hydroxyethyl starch is used and coupled directly to proteins using ethyldimethylaminopropyl carbodiimide (EDC) activation, or a non-oxidized hydroxyethyl starch is used and coupled directly, i.e. without linking compound to a protein forming a Schiff's base which is subsequently reduced to the respective amine. Thus, the working examples of WO 03/074087 do not disclose a single conjugate comprising hydroxyethyl starch, the protein, and one or more linker molecules. Additionally, WO 03/074087 does not contain any information as to a linking compound comprising an aminooxy, i.e. a hydroxylamino group.

Consequently, it is an object of the present invention to provide polypeptide derivatives, especially erythropoietin derivatives, having a high biological activity in vivo which can be easily produced and at reduced costs.

Therefore, it is another object of the present invention to provide conjugates of erythropoietin and hydroxyethyl starch with improved specific in vivo activity.

It is yet another object of the present invention to provide a method for preparing these conjugates of erythropoietin and hydroxyethyl starch with improved specific in vivo activity.

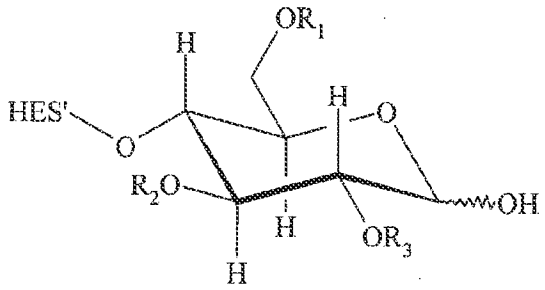
It is still another object of the present invention to provide a method for improving the specific in vivo activity of conjugates of erythropoietin and hydroxyethyl starch by changing the characteristics of the hydroxyethyl starch used for the production of the conjugates.

Therefore, the present invention relates to a conjugate of erythropoietin and hydroxyethyl starch, comprising a homobifunctional crosslinking compound having two hydroxylamino groups, one of which is covalently linked to a carbohydrate moiety of the erythropoietin and one of which is covalently linked to the hydroxyethyl starch, wherein the hydroxyethyl starch has a mean molecular weight of at least 40 kD and a degree of substitution of at least 0.6.

Furthermore, the present invention also relates to a method of producing a conjugate of erythropoietin and hydroxyethyl starch, said method comprising reacting the hydroxyethyl starch with a homobifunctional crosslinking compound having two hydroxylamino groups to give a hydroxylamino functionalized hydroxyethyl starch derivative, and reacting the hydroxylamino group of said derivative with a carbohydrate moiety of the erythropoietin, wherein the hydroxyethyl starch has a mean molecular weight of at least 40 kD and a degree of substitution of at least 0.6.

Furthermore, the present invention also relates to a conjugate obtainable by the method according to the invention.

In the context of the present invention, the term "hydroxyethyl starch" (HES) refers to a starch derivative which has been substituted by at least one hydroxyethyl group. A preferred hydroxyethyl starch of the present invention has a structure according to formula (I)



(I)

wherein the reducing end of the starch molecule is shown in the non-oxidized form and the terminal saccharide unit is shown in the acetal form which, depending on e.g. the solvent, may be in equilibrium with the aldehyde form. In formula (I), the saccharide ring described explicitly and the residue denoted as HES' together represent the preferred hydroxyethyl starch molecule. The other saccharide ring structures comprised in HES' may be the same as or different from the explicitly described saccharide ring.

The term "hydroxyethyl starch" as used in the present invention is not limited to compounds where the terminal carbohydrate moiety comprises hydroxyethyl groups R_1, R_2, and/or R_3 as depicted, for the sake of brevity, in formula (I), but also refers to compounds in which at least one hydroxy group present anywhere, either in the terminal

carbohydrate moiety and/or in the remaining part of the starch molecule, HES', is substituted by a hydroxyethyl group R_1, R_2, or R_3.

The at least one hydroxyethyl group comprised in HES may contain two or more hydroxy groups. According to a preferred embodiment, the at least one hydroxyethyl group comprised in HES contains one hydroxy group.

The expression "hydroxyethyl starch" also includes derivatives wherein the ethyl group is mono- or polysubstituted. In this context, it is preferred that the ethyl group is substituted with a halogen, especially fluorine, or with an aryl group. Furthermore, the hydroxy group of a hydroxyethyl group may be esterified or etherified.

Techniques for the esterification of starch are known in the art (see e.g. Klemm D. et al, Comprehensive Cellulose Chemistry Vol. 2, 1998, Whiley-VCH, Weinheim, New York, especially chapter 4.4, Esterification of Cellulose (ISBN 3-527-29489-9).

As far as the residues R_1, R_2 and R_3 according to formula (I) are concerned, there are no specific limitations. According to a preferred embodiment, R_1, R_2 and R_3 are independently hydrogen or a 2-hydroxyethyl group.

Hydroxyethyl starch (HES) is a derivative of naturally occurring amylopectin and is degraded by alpha-amylase in the body. HES is a substituted derivative of the carbohydrate polymer amylopectin, which is present in corn starch at a concentration of up to 95 % by weight. HES exhibits advantageous biological properties and is used as a blood volume replacement agent and in hemodilution therapy in the clinics (Sommermeyer et al., 1987, Krankenhauspharmazie, 8(8), 271-278; and Weidler et al., 1991, Arzneim.-Forschung/Drug Res., 41, 494-498).

Amylopectin consists of glucose moieties, wherein in the main chain alpha-1,4-glycosidic bonds are present and at the branching sites alpha-1,6-glycosidic bonds are found. The physical-chemical properties of this molecule are mainly determined by the type of glycosidic bonds. Due to the nicked alpha-1,4-glycosidic bond, helical structures with about six glucose-monomers per turn are produced. The physico-chemical as well as the biochemical properties of the polymer can be modified via substitution. The introduction

of a hydroxyethyl group can be achieved via alkaline hydroxyethylation. By adapting the reaction conditions it is possible to exploit the different reactivity of the respective hydroxy group in the unsubstituted glucose monomer with respect to a hydroxyethylation. Owing to this fact, the skilled person is able to influence the substitution pattern to a limited extent.

HES is mainly characterized by the molecular weight distribution and the degree of substitution. There are two possibilities of describing the substitution degree:

1. The degree of substitution can be described relatively to the portion of substituted glucose monomers with respect to all glucose moieties.
2. The degree of substitution can be described as the molar substitution, wherein the number of hydroxyethyl groups per glucose moiety are described.

In the context of the present invention, the degree of substitution, denoted as DS, relates to the molar substitution, as described above (see also Sommermeyer et al., 1987, Krankenhauspharmazie, 8(8), 271-278, as cited above, in particular p. 273).

HES preparations are present as polydisperse compositions, wherein each molecule differs from the other with respect to the polymerisation degree, the number and pattern of branching sites, and the substitution pattern. HES is therefore a mixture of compounds with different molecular weight. Consequently, a particular HES solution is determined by average molecular weight with the help of statistical means. In this context, M_n is calculated as the arithmetic mean depending on the number of molecules. Alternatively, M_w (or MW), the weight mean, represents a unit which depends on the mass of the HES.

In the context of the present invention, the hydroxyethyl starch has a mean molecular weight (weight mean) of at least about 40 kD and degree of substitution DS of at least about 0.6.

According to more preferred embodiments of the present invention, hydroxyethyl starch has a mean molecular weight (weight mean) of at least about 40 kD and degree of substitution DS of greater than about 0.6 or a mean molecular weight (weight mean) of greater than about 40 kD and degree of substitution DS of at least about 0.6. According to an even more preferred embodiment, hydroxyethyl starch has a mean molecular weight (weight mean) of greater than about 40 kD and degree of substitution DS of greater than about 0.6.

According to a still further preferred embodiment of the present invention, the hydroxyethyl starch has a mean molecular weight (weight mean) of at least about 50 or 100 kD and degree of substitution DS of at least about 0.7, more preferably having a mean molecular weight (weight mean) of about 50 or 100 kD and degree of substitution DS of at least about 0.7 or having a mean molecular weight (weight mean) of at least about 50 or 100 kD and degree of substitution DS of about 0.7 and even more preferably having a mean molecular weight (weight mean) of about 50 or 100 kD and degree of substitution DS of about 0.7.

Therefore, the present invention also relates to a conjugate and a method as described above, wherein the hydroxyethyl starch has a mean molecular weight of at least 50 kD and a degree of substitution of at least 0.7.

Accordingly, the present invention also relates to a conjugate and a method as described above, wherein the hydroxyethyl starch has a mean molecular weight of about 50 kD and a degree of substitution of about 0.7.

According to another embodiment of the present invention, the hydroxyethyl starch has a mean molecular weight (weight mean) of at least about 50 kD and degree of substitution DS of at least about 0.8, more preferably having a mean molecular weight (weight mean) of about 50 kD and degree of substitution DS of at least about 0.8 or having a mean molecular weight (weight mean) of at least about 50 kD and degree of substitution DS of about 0.8 and even more preferably having a mean molecular weight (weight mean) of about 50 kD and degree of substitution DS of about 0.8.

Further described is a hydroxyethyl starch having a mean molecular weight (weight mean) of at least about 120 kD and degree of substitution DS of at least about 0.6, such as a mean molecular weight of about 120 kD and degree of substitution DS of at least about 0.6, or a mean molecular weight of about 120 kD and degree of substitution DS of at least about 0.7, or a mean molecular weight of about 120 kD and degree of substitution DS of at least about 0.8, or a mean molecular weight of about 120 kD and degree of substitution DS of at least about 0.9, or a mean molecular weight of about 130 kD and degree of substitution DS of at least about 0.6, or a mean molecular weight of about 130 kD and degree of substitution DS of at least about 0.7, or a mean molecular weight of about 130 kD and

degree of substitution DS of at least about 0.8, or a mean molecular weight of about 130 kD and degree of substitution DS of at least about 0.9, or a mean molecular weight of about 140 kD and degree of substitution DS of at least about 0.6, or a mean molecular weight of about 140 kD and degree of substitution DS of at least about 0.7, or a mean molecular weight of about 140 kD and degree of substitution DS of at least about 0.8, or a mean molecular weight of about 140 kD and degree of substitution DS of at least about 0.9.

Therefore, the present invention also relates to a conjugate and a method as described above, wherein the hydroxyethyl starch has a mean molecular weight of about 130 kD and a degree of substitution of about 0.6 or of about 0.7 or of about 0.8 or of about 0.9.

The term "about 40 kD" as used in the context of the present relates to a mean molecular weight in the range of from 38 kD to 42 kD, more preferably in the range of from 39 kD to 41 kD.

The term "about 50 kD" as used in the context of the present relates to a mean molecular weight in the range of from 48 kD to 52 kD, more preferably in the range of from 49 kD to 51 kD.

The term "about 120 kD" as used in the context of the present relates to a mean molecular weight in the range of from 116 kD to 124 kD, more preferably in the range of from 118 kD to 122 kD.

The term "about 130 kD" as used in the context of the present relates to a mean molecular weight in the range of from 126 kD to 134 kD, more preferably in the range of from 128 kD to 132 kD.

The term "about 140 kD" as used in the context of the present relates to a mean molecular weight in the range of from 136 kD to 144 kD, more preferably in the range of from 138 kD to 142 kD.

The term "about 0.6" as used in the context of the present with regard to DS relates to a degree of substitution in the range of greater than 0.55 to 0.65, more preferably in the range of from 0.58 to 0.62.

The term "about 0.7" as used in the context of the present with regard to DS relates to a degree of substitution in the range of greater than 0.65 to 0.75, more preferably in the range of from 0.68 to 0.72.

The term "about 0.8" as used in the context of the present with regard to DS relates to a degree of substitution in the range of greater than 0.75 to 0.85, more preferably in the range of from 0.78 to 0.82.

The term "about 0.9" as used in the context of the present with regard to DS relates to a degree of substitution in the range of greater than 0.85 to 0.95, more preferably in the range of from 0.88 to 0.92.

As far as the ratio of C_2 : C_6 substitution of the hydroxyethyl starch is concerned, said substitution is preferably in the range of from 2 to 20, more preferably in the range of from 2 to 15 and even more preferably in the range of from 3 to 12.

The term "mean molecular weight" as used in the context of the present invention relates to the weight as determined according the LALLS-(low angle laser light scattering)-GPC method as described in Sommermeyer et al., 1987, Krankenhauspharmazie, 8(8), 271-278; and Weidler et al., 1991, Arzneim.-Forschung/Drug Res., 41, 494-498. For mean molecular weights of 10 kD and smaller, additionally, the calibration was carried out classically with a standard which had been previously qualified by LALLS-GPC.

The EPO used according to the present invention can be of any human (see e.g. Inoue, Wada, Takeuchi, 1994, An improved method for the purification of human erythropoietin with high in vivo activity from the urine of anemic patients, Biol Pharm Bull. 17(2), 180-4; Miyake, Kung, Goldwasser, 1977, Purification of human erythropoietin., J Biol Chem., 252(15), 5558-64) or another mammalian source and can be obtained by purification from naturally occurring sources like human kidney, embryonic human liver or animal, preferably monkey kidney. Furthermore, the expression "erythropoietin" or "EPO"

encompasses also an EPO variant wherein one or more amino acids (e.g. 1 to 25, preferably 1 to 10, more preferred 1 to 5, most preferred 1 or 2) have been exchanged by another amino acid and which exhibits erythropoietic activity (see e.g. EP 640 619 B1). The measurement of erythropoietic activity is described in the art (for measurement of activity in vitro see e.g. Fibi et al.,1991, Blood, 77, 1203 ff; Kitamura et al, 1989, J. Cell Phys., 140, 323-334; for measurement of EPO activity in vivo see Ph. Eur. 2001, 911-917; Ph. Eur. 2000, 1316 Erythropoietini solutio concentrata, 780- 785; European Pharmacopoeia (1996/2000); European Pharmacopoeia, 1996, Erythropoietin concentrated solution, Pharmaeuropa., 8, 371-377; Fibi, Hermentin, Pauly, Lauffer, Zettlmeissl., 1995, N- and O-glycosylation muteins of recombinant human erythropoietin secreted from BHK-21 cells, Blood, 85(5), 1229-36; (EPO and modified EPO forms were injected into female NMRI mice (equal amounts of protein 50 ng/mouse) at day 1, 2 and 3 blood samples were taken at day 4 and reticulocytes were determined)). Further publications where tests for the measurement of the activity of EPO are Barbone, Aparicio, Anderson, Natarajan, Ritchie, 1994, Reticulocytes measurements as a bioassay for erythropoietin, J. Pharm. Biomed. Anal., 12(4), 515-22; Bowen, Culligan, Beguin, Kendall, Villis, 1994, Estimation of effective and total erythropoiesis in myelodysplasia using serum transferrin receptor and erythropoietin concentrations, with automated reticulocyte parameters, Leukemi, 8(1), 151-5; Delorme, Lorenzini, Giffin, Martin, Jacobsen, Boone, Elliott, 1992, Role of glycosylation on the secretion and biological activity of erythropoietin, Biochemistry, 31(41), 9871-6; Higuchi, Oh-eda, Kuboniwa, Tomonoh, Shimonaka, Ochi, 1992;Role of sugar chains in the expression of the biological activity of human erythropoietin, J. Biol. Chem., 267(11), 7703-9; Yamaguchi, Akai, Kawanishi, Ueda, Masuda, Sasaki, 1991, Effects of site-directed removal of N-glycosylation sites in human erythropoietin on its production and biological properties, J. Biol. Chem., 266(30), 20434-9; Takeuchi, Inoue, Strickland, Kubota, Wada, Shimizu, Hoshi, Kozutsumi, Takasaki, Kobata, 1989, Relationship between sugar chain structure and biological activity of recombinant human erythropoietin produced in Chinese hamster ovary cells, Proc. Natl. Acad. Sci. USA, 85(20), 7819-22; Kurtz, Eckardt, 1989, Assay methods for erythropoietin, Nephron., 51(1), 11-4 (German); Zucali, Sulkowski, 1985, Purification of human urinary erythropoietin on controlled-pore glass and silicic acid, Exp. Hematol., 13(3), 833-7; Krystal, 1983, Physical and biological characterization of erythroblast enhancing factor (EEF), a late acting erythropoetic stimulator in serum distinct from erythropoietin, Exp. Hematol., 11(1), 18-31.

According to a preferred embodiment of the present invention, EPO is recombinantly produced. This includes the production in eukaryotic or prokaryotic cells, preferably mammalian, insect, yeast, bacterial cells or in any other cell type which is convenient for the recombinant production of EPO. Furthermore, the EPO may be expressed in transgenic animals (e.g. in body fluids like milk, blood, etc.), in eggs of transgenic birds, especially poultry, preferred chicken, or in transgenic plants.

Therefore, the present invention also relates to a conjugate and a method as described above, wherein EPO is recombinantly produced.

The recombinant production of a polypeptide is known in the art. In general, this includes the transfection of host cells with an appropriate expression vector, the cultivation of the host cells under conditions which enable the production of the polypeptide and the purification of the polypeptide from the host cells. For detailled information see e.g. Krystal, Pankratz, Farber, Smart, 1986, Purification of human erythropoietin to homogeneity by a rapid five-step procedure, Blood, 67(1), 71-9; Quelle, Caslake, Burkert, Wojchowski, 1989, High-level expression and purification of a recombinant human erythropoietin produced using a baculovirus vector, Blood, 74(2), 652-7; EP 640 619 B1 and EP 668 351 B1.

In a preferred embodiment, the EPO has the amino acid sequence of human EPO (see EP 148 605 B2). Therefore, the present invention also relates to a conjugate and a method as described above, wherein EPO has the amino acid sequence of human EPO.

The EPO may comprise one or more carbohydrate side chains (preferably 1-4, preferably 4) attached to the EPO via N- and/ or O-linked glycosylation, i.e. the EPO is glycosylated. Usually, when EPO is produced in eukaryotic cells, the polypeptide is posttranslationally glycosylated. Consequently, the carbohydrate side chains may have been attached to the EPO during biosynthesis in mammalian, especially human, insect or yeast cells. The structure and properties of glycosylated EPO have been extensively studied in the art (see EP 428 267 B1; EP 640 619 B1; Rush, Derby, Smith, Merry, Rogers, Rohde, Katta, 1995, Microheterogeneity of erythropoietin carbohydrate structure, Anal Chem., 67(8), 1442-52;

Takeuchi, Kobata, 1991, Structures and functional roles of the sugar chains of human erythropoietins, Glycobiology, 1(4), 337-46 (Review).

Therefore, the present invention also relates to a conjugate and a method as described above, wherein the carbohydrate moiety is comprised in a carbohydrate side chain which was attached to the erythropoietin via N- and/ or O-linked glycosylation, the erythropoietin comprising at least one carbohydrate side chain.

Thus, the present invention also relates to a conjugate and a method as described above, wherein the at least one carbohydrate side chain was attached to the erythropoietin during the production of the erythropoietin in mammalian, especially human cells, insect cells, yeast cells, transgenic animals or transgenic plants

According to the present invention, a hydroxylamino group of the crosslinking compound is linked to a carbohydrate moiety of the erythropoietin. In the context of the present invention, the term "carbohydrate moiety" refers to hydroxyaldehydes or hydroxyketones as well as to chemical modifications thereof (see Römpp Chemielexikon, Thieme Verlag Stuttgart, Germany, 9th edition 1990, Volume 9, pages 2281-2285 and the literature cited therein). Furthermore, it also refers to derivatives of naturally occuring carbohydrate moieties like glucose, galactose, mannose, sialic acid and the like. The term also includes chemically oxidized naturally occuring carbohydrate moieties wherein the ring structure has been opened.

Thus, in the context of the present invention, the hydroxylamino group is linked to an aldehyde group or a keto group of the carbohydrate moiety, especially preferably to an aldehyde group of the carbohydrate moiety.

Therefore, the present invention also relates to a method and a conjugate as described above, wherein the crosslinking compound is linked via an oxime linkage to the hydroxyethyl starch and to the carbohydrate moiety of the erythropoietin.

The carbohydrate moiety may be linked directly to the EPO polypeptide backbone. Preferably, the carbohydrate moiety is part of a carbohydrate side chain. In this case, further carbohydrate moieties may be present between the carbohydrate moiety to which

the hydroxylamino group is linked and the EPO polypeptide backbone. More preferably, the carbohydrate moiety is the terminal moiety of a carbohydrate side chain.

In a more preferred embodiment, the hydroxylamino group is linked to a galactose residue of a carbohydrate side chain, preferably the terminal galactose residue of a carbohydrate side chain. This galactose residue can be made available for conjugation by removal of terminal sialic acids, followed by oxidation.

In a still further preferred embodiment, the hydroxylamino group is linked to a preferably oxidized sialic acid residue of a carbohydrate side chains, preferably the terminal sialic acid residue of a carbohydrate side chain.

The EPO may comprise one or more carbohydrate side chains attached to the EPO via N- and/ or O-linked glycosylation, i.e. the EPO is glycosylated. Unsually, when EPO is produced in eukaryotic cells, the polypeptide is posttranslationally glycosylated. Consequently, the carbohydrate side chains may have been attached to the EPO during production in mammalian, especially human, insect or yeast cells, which may be cells of a transgenic animal, either extracted from the animal or still in the animal.

These carbohydrate side chains may have been chemically or enzymatically modified after the expression in the appropriate cells, e.g. by removing or adding one or more carbohydrate moieties (see e.g. Dittmar, Conradt, Hauser, Hofer, Lindenmaier, 1989, Advances in Protein design; Bloecker, Collins, Schmidt, and Schomburg eds., GBF-Monographs, 12, 231-246, VCH Publishers, Weinheim, New York, Cambridge)

According to an especially preferred embodiment of the present invention, the carbohydrate moiety is the terminal moiety of the carbohydrate side chain.

Consequently, in a preferred embodiment, the HES reacted with the crosslinking compound is linked to carbohydrate chains linked to N- and/ or O-glycosylation sites of EPO.

It is also included within the present invention that the EPO contains a further carbohydrate moiety or further carbohydrate moieties to which the hydroxylamino group of a

crosslinking compound is linked to. Techniques for attaching carbohydrate moieties to polypeptides, either enzymatically or by genetic engineering, followed by expression in appropriate cells, are known in the art (Berger, Greber, Mosbach, 1986, Galactosyltransferase-dependent sialylation of complex and endo-N-acetylglucosaminidase H-treated core N-glycans in vitro, FEBS Lett., 203(1), 64-8; Dittmar, Conradt, Hauser, Hofer, Lindenmaier, 1989, Advances in Protein design; Bloecker, Collins, Schmidt, and Schomburg eds., GBF-Monographs, 12, 231-246, VCH Publishers, Weinheim, New York, Cambridge).

In a preferred embodiment of the method of the invention, the carbohydrate moiety is oxidized in order to be able to react with the hydroxylamino group. This oxidation can be performed either chemically or enzymatically.

Methods for the chemical oxidation of carbohydrate moieties of polypeptides are known in the art and include the treatment with periodate (Chamow et al., 1992, J. Biol. Chem., 267, 15916-15922).

By chemically oxidizing, it is principally possible to oxidize any carbohydrate moiety, being terminally positioned or not. However, by choosing mild conditions (e.g., 1 mM periodate, 0 °C in contrast to harsh conditions, e.g.: 10 mM periodate 1h at room temperature), it is possible to preferably oxidize the terminal sialic acid of a carbohydrate side chain.

Alternatively, the carbohydrate moiety may be oxidized enzymatically. Enzymes for the oxidation of the individual carbohydrate moieties are known in the art, e.g. in the case of galactose the enzyme is galactose oxidase.

If it is intended to oxidize terminal galactose moieties, it will be eventually necessary to partially or completely remove terminal sialic acids if the EPO has been produced in cells capable of attaching sialic acids to carbohydrate chains, e.g. in mammalian cells or in cells which have been genetically modified to be capable of attaching sialic acids to carbohydrate chains. Chemical or enzymatic methods for the removal of sialic acids are known in the art (Chaplin and Kennedy (eds.), 1996, Carbohydrate Analysis: a practical

approach, especially Chapter 5 Montreuill, Glycoproteins, pages 175-177; IRL Press Practical approach series (ISBN 0-947946-44-3)).

However, it is also included within the present invention that the carbohydrate moiety to which the hydroxylamino group is linked to is suitably attached to the EPO. In the case it is preferred to attach galactose. This can be achieved by the means of galactosyltransferase. The methods are known in the art (Berger, Greber, Mosbach, 1986, Galactosyltransferase-dependent sialylation of complex and endo-N-acetylglucosaminidase H-treated core N-glycans in vitro, FEBS Lett., 203(1), 64-8).

In a most preferred embodiment of the present invention, at least one terminal saccharide unit of the EPO is oxidized, preferably galactose, most preferably sialic acid, of the one or more carbohydrate side chains of the EPO, optionally after partial or complete (enzymatic and/or chemical) removal of the terminal sialic acid, if necessary.

Consequently, the modified HES is conjugated to the oxidized terminal saccharide unit of the carbohydrate chain, preferably sialic acid.

Furthermore, the modified HES may be preferably conjugated to a terminal sialic acid, which is still more preferably oxidized.

Therefore, the present invention also relates to a conjugate and a method as described above, wherein the reaction is carried out at a temperature of from 20 to 25 °C.

Therefore, the present invention also relates to a conjugate and a method as described above, wherein the carbohydrate moiety is an oxidized terminal saccharide unit of a carbohydrate side chain the erythropoietin, preferably an oxidized sialic acid.

Therefore, the present invention also relates to a conjugate and a method as described above, wherein the terminal saccharide unit was oxidized after partial or complete, enzymatic and/ or chemical removal of the terminal sialic acid.

Therefore, the present invention also relates to a conjugate and a method as described above, wherein the terminal saccharide unit is galactose.

Therefore, the present invention also relates to a conjugate and a method as described above, wherein the carbohydrate moiety is comprised in a carbohydrate side chain of the erythropoietin which was attached to the erythropoietin via N- and/ or O-linked glycosylation during its production in mammalian, especially human cells, insect cells, or yeast cells.

According to the present invention, a hydroxylamino group of a crosslinking compound is covalently linked to the carbohydrate moiety of EPO.

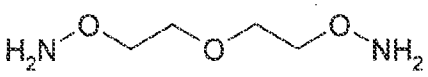
The term "hydroxylamino group" as used in the context of the present invention relates to a functional group according to formula -O-NH-R or -NH-O-R where R is hydrogen or an optionally suitably substituted alkyl residue, aryl residue, alkaryl residue or aralky residue. In case the hydroxylamino group -O-NH-R, R is preferably hydrogen and alkyl such as methyl, ethyl, propyl and butyl, more preferably hydrogen and methyl an especially preferably hydrogen. In case the hydroxylamino group -O-NH-R, R is preferably hydrogen and alkyl such as methyl, ethyl, propyl and butyl, more preferably hydrogen and methyl an especially preferably methyl. According to an especially preferred embodiment of the present invention, the hydroxylamino group -O-NH-R and R is hydrogen.

The crosslinking compound according to the invention may comprise the same or different hydroxlyamino groups, preferably the same hydroxylamino groups such as two methylaminooxy groups or two aminooxy groups or two methoxyamino groups.

The two hydroxylamino groups, comprised in the crosslinking compound according to the present invention, may be separated by a suitable spacer. Among others, the spacer may be an optionally substituted, linear, branched and/or cyclic hydrocarbon residue. Generally, the hydrocarbon residue has up to 60, preferably up to 40, more preferably up to 20, more preferably up to 10, more preferably up to 6 and especially preferably up to 4 carbon atoms. If heteroatoms are present, the spacer comprises generally from 1 to 20, preferably from 1 to 8, more preferably 1 to 6, more preferably 1 to 4 and especially preferably from 1 to 2 heteroatoms. As heteroatom, S, N or O are preferred, O being especially preferred. The hydrocarbon residue may comprise an optionally branched alkyl chain or an aryl group or a cycloalkyl group having, e.g., from 5 to 7 carbon atoms, or be an aralkyl group, an alkaryl group where the alkyl part may be a linear and/or cyclic alkyl group. According

to an even more preferred embodiment of the present invention, the hydroxylamino groups are separated by a linear hydrocarbon chain having 4 carbon atoms. According to another preferred embodiment of the present invention, the functional groups are separated by a linear hydrocarbon chain having 4 carbon atoms and at least one, preferably one heteroatom, particularly preferably an oxygen atom. Particularly preferred is a spacer according to formula -CH_2-CH_2-O-CH_2-CH_2-.

Therefore, preferred crosslinking compounds according to the present invention are



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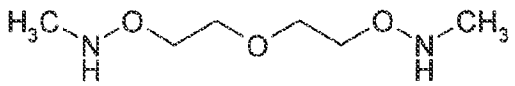

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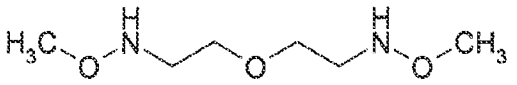
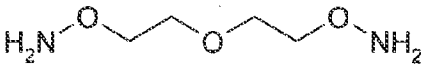


with



being especially preferred.

The conjugate of the present invention may exhibit essentially the same in-vitro biological activity as recombinant native EPO, since the in-vitro biological activity only measures binding affinity to the EPO receptor. Methods for determining the in-vitro biological activity are known in the art (see, e.g., Fibi et al., 1991, Blood, 77, 1203 ff; Kitamura et al, 1989, J. Cell Phys., 140, 323-334).

Furthermore, the conjugate of the present invention may exhibits a greater in vivo activity than the EPO used as a starting material for conjugation (non-conjugated EPO). Methods for determining the in vivo biological activity are known in the art (see, e.g., Example above). Furthermore, assays for the determination of in vivo activity are given in Example 7.

The conjugate may exhibit an in vivo activity of 110 to 500 %, preferably 200 to 500 %, more preferably 300 % to 500 %, more preferred 400 % to 500 % such as 450 % to 500 %

or 450 to 490 % or 450 % to 480 % or 450 % to 470 %, the in vivo activity of the non-modified EPO set as 100 %.

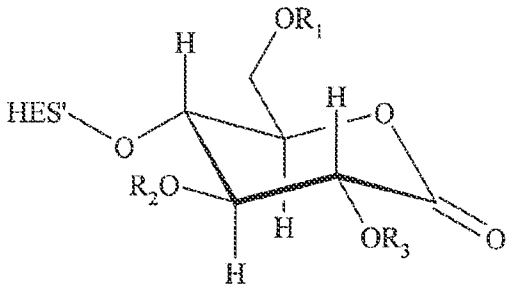
The high in vivo biological activity of the conjugate according to the present invention mainly results from the fact that the conjugate remains longer in the circulation than the non-conjugated EPO, because it is less recognized by the removal systems of the liver and because renal clearance is reduced due to the higher molecular weight. Methods for the determination of the in vivo half life time of EPO in the circulation are known in the art (Sytkowski, Lunn, Davis, Feldman, Siekman, 1998, Human erythropoietin dimers with markedly enhanced in vivo activity, Proc. Natl. Acad. Sci. USA, 95(3), 1184-8).

Consequently, it is a great advantage of the present invention that a conjugate is provided that may be administered less frequently than the EPO preparations commercially available at present. While standard EPO preparations have to be administered at least every 3 days, the conjugate of the invention is preferably administered twice a week, more preferably once a week.

According to the first step of the method of the present invention, HES is reacted with a hydroxylamino group, preferably with the group -O-NH_2 of the crosslinking compound.

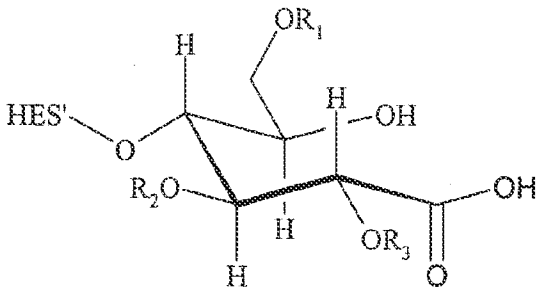
In general, it is possible to react the hydroxylamino group with any suitable functional group of HES. According to especially preferred embodiments of the present invention, the hydroxylamino group is reacted with the reducing end of HES, which is oxidized or which is not oxidized.

In case the hydroxylamino group is reacted with the reducing end of HES in its oxidized form, HES is preferably used having a structure according to formula (IIa)



(IIa)

and/or according to formula (IIb)



(IIb)

The oxidation of the reducing end of the polymer, preferably hydroxyethyl starch, may be carried out according to each method or combination of methods which result in compounds having the above-mentioned structures (IIa) and/or (IIb). This oxidation is preferably carried out using an alkaline iodine solution as described, e.g., in DE 196 28 705 A1 the respective contents of which (example A, column 9, lines 6 to 24) is incorporated herein by reference.

According to an especially preferred embodiment of the present invention, HES is employed with its reducing end in the non-oxidized form, i.e. HES according to formula (I).

Therefore, the present invention also relates to a method and a conjugate as described above, wherein the crosslinking compound is reacted with HES, HES being employed with its reducing end in the non-oxidized form.

It is possible to react the crosslinking compound with HES in any suitable solvent. According to an especially preferred embodiment, this reaction is carried out in an aqueous medium.

Therefore, the present invention also relates to a method and a conjugate as described above, wherein the hydroxyethyl starch is reacted with the homobifunctional crosslinking compound in an aqueous medium.

The term "aqueous medium" as used in the context of the present invention relates to a solvent or a mixture of solvents comprising water in the range of from at least 10 % per weight, more preferably at least 20 % per weight, more preferably at least 30 % per weight,